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Full Length Article

Morphological, Pathogenic and Molecular Characterization of *Lasiodiplodia theobromae*: A Causal Pathogen of Black Rot Disease on Kenaf Seeds in Malaysia

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Abstract

Kenaf (*Hibiscus cannabinus*) is a fibre crop grown in Malaysia as a substitute crop for tobacco. Previous study have recorded that kenaf has been infected by various genera of seed-borne pathogen include *Fusarium*, *Synnematium*, *Alternaria*, *Colletotrichum* and *Botrytis*. Seed-borne disease affects and actively attacks seeds and may be harmful. *Lasiodiplodia theobromae* is a seed-borne fungal pathogen that infects a variety of crop seeds. Studies on the isolation of seed-borne fungi on kenaf seed have revealed that *L. theobromae* causes black rot disease on kenaf seeds. *L. theobromae* was successfully isolated from kenaf seeds on an agar plate and a blotter. *L. theobromae* was isolated frequently from infected seeds and identified based on its cultural and morphological characteristics. The fungus sequence was analysed using molecular technique (ITS-rDNA amplification). A pathogenicity test was used to confirm that *L. theobromae* caused blackening of the seeds and reduced the germination against a control treatment in potato Dextrose Agar (PDA) medium. To our knowledge, this study is the first to confirm that *L. theobromae* is the causal agent of black rot on kenaf seed in Malaysia. © 2016 Friends Science Publishers

Keywords: *Lasiodiplodia theobromae*; Kenaf; Seed-borne fungal pathogen; ITS-rDNA amplification

Introduction

Kenaf (*Hibiscus cannabinus* L.) is a common warm season annual fibre plant found in tropical and subtropical Africa and Asia. Kenaf can be processed into various products such as building materials, furniture, clothing, car component, biofuel and animal feed (Dempsey, 1975). In Malaysia, kenaf has been cultivated as a substitute crop for tobacco because of the eventual decline in imported prices (Toreksulong, 2010). Kenaf has the potential to become one of the main crop plants in Malaysia and has been treated as a new commodity and source of growth in the country (Mohd Sahwahid *et al.*, 2012).

Even though kenaf is resistant to many types of infectious diseases, it has been infected by some pathogens including fungi, bacteria, nematodes and viruses. Previous studies on kenaf diseases demonstrated pathogens such as *Rhizoctonia solani*, *Sclerotium rolfsii*, *Phytophthora parasitica* (Dempsey, 1975), *Ralstonia solanacearum* and nematodes (Mat Daham *et al.*, 2005) have been associated with kenaf disease infection.

Seed-borne disease pathogens actively attack seeds and may be harmful. Seeds can be infected by pathogens that colonise the seeds both externally or internally. The infection of seeds by microorganisms (e.g., fungus, bacteria and virus) has adverse effects on seeds, such germination losses. Germination loss due to seed infections may indirectly decrease crop yields by interrupting radical emergence of the seed coat in pre-emergence stage, thereby reducing the overall crop stand. Infection occuring after the post-emergence (i.e., after seedlings emerge from the soil) include root rot, cotyledon rot and basal stem rot (Mithal and Mathur, 2003). Swart and Tarekegn (2007) reported that *Fusarium verticilloides* causes damping off on kenaf in South Africa. The damage of seedlings and the interruption of plant development will result from systemic or local infections of seed-borne pathogens in later stages (Bateman and Kwasna, 1999; Khazanda *et al.*, 2002).

Botryosphaeriaceae is common and widely distributed in many plants including monocotyledons, dicotyledons and gymnosperm hosts (von Arx and Muller, 1975). Fungi that are grouped together as *Botryosphaeria* have been reported to cause various symptoms of disease including shoot blight, dieback stem canker, seed capsule abortion, seed and fruit rots (Webb, 1983; Smith *et al.*, 1996; Roux and Wingfield, 1997; Roux *et al.*, 2001; Gezahgne *et al.*, 2003; Alves *et al.*, 2004 and Gezahgne, 2004). Other diseases include dieback (Sharma and Sankaran, 1988), post-harvest disease of fruits (Mascarenhas *et al.*, 1995; Gupta *et al.*, 1999; Ploetz, 2003), gummosis (Li *et al.*, 1995; Muniz *et al.*, 2011), stem canker (Sharma *et al.*, 1984; Sánchez *et al.*, 2003), witches' broom and black seed rot (Fraedrich *et al.*,

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1994; Bankole *et al.*, 1999; Gure *et al.*, 2005). In Malaysia, *Botryosphaeria* spp. is important because it cause several plant diseases.

Several genera of pathogenic fungi have been reported on kenaf seeds by Yusen *et al.* (2003) including *Fusarium*, *Synnematium*, *Alternaria*, *Colletotrichum* and *Botrytis*. The impact of potential fungal pathogens on kenaf needs to be investigated in order to formulate suitable disease control protocols. Therefore, this study was designed to isolate and characterise seed-borne fungi on kenaf seeds that may affect their germination.

Materials and Methods

Isolation of Fungal Pathogen from Kenaf Seeds

Seeds were collected from a seed storage located at the National Tobacco and Kenaf Board (NTKB) in Kangar Perlis, Malaysia. Isolation was accomplished using two methods: direct plating and with a blotter. Two hundred seeds were used for each methods. The seeds were placed on sterile moistened filter paper for the blotter. Sterile surface seeds were placed in water agar. All seeds were incubated for 7 days. After the incubation period, the fungus was subcultured on potato dextrose agar (PDA).

Pathogenicity Test

Fungus was tested for pathogenicity using artificial inoculation in PDA. The disease assessment was conducted to observe the effect of fungus on the seeds. After the incubation period, data were recorded regarding the seed germination, the pre-emergence damping-off and the seedling survival.

Cultural and Morphological Characterization

The morphology of conidia was examined using carnation leaf embedded in the PDA. Sterile fragments of carnation leaves were sprinkled into the PDA. Tips of mycelia were placed into the PDA content with carnation leaves and were incubated for approximately 30 days at room temperature (28±2°C). After the fungus was germinated, conidia were collected and observed under a light microscope. The conidial morphology was studied and their shape, colour and presence of septation were recorded. The conidia were compared to a dichotomous key obtained from Burgess *et al.* (2006).

DNA Extraction, ITS-rDNA Amplification and Sequencing of Fungal Pathogen

Pure cultures of isolated fungus were transferredi into the PDA (5 mm) and were incubated for seven days before extracting their DNA. DNA extraction was accomplished using the CTAB method suggested by (Edward *et al.*, 1991) with slight modifications. The PCR amplification of the ITS-rDNA region of fungal isolates was performed using 2 oligonucleotide primers: a forward primer (ITS 1; 5’-TCCGTAGGTGAACCTGCGG-3’) and a reverse primer, (ITS 4; 5’-TCCTCCGCTTAATTGATATGC-3’), have been described by White *et al.* (1990). PCR was perfomerd in a 25 µL reaction containing 10 mM Green Taq Fermentas PCR Master Mix, 0.4 µM of ITS 1, 0.4 µM of ITS 4, 3 µL of the DNA template and 7.5 µL of nuclease-free water. The thermal cycle conditions were as followed: 1 cycle of initial denaturation at 96°C for 3 min, 35 cycle at denaturing temperature 95°C for 3 min, annealing temperature 56°C for 1 min and extension temperature 72°C for 10 min and final extension at 72°C for 5 min. The PCR product was visualised using a 2% agarose gel in a 1X TAE buffer and was purified using a Fermentas GeneJet PCR Product Purification Kit. The nucleotide sequence was identified using a Basic Local Alignment Search Tool (BLAST). A phylogenetic tree was constructed by using neighbour joining method with default values (MEGA software version 4).

Experimental Design and Statistical Analysis

All the experiments were conducted following a completely randomized design (CRD), and data were analysed using an analysis of variance (ANOVA) with duncan’s multiple range test.

Results

Isolation of Fungal Pathogen from Kenaf Seeds

From the 200 seeds used for each isolation method, 10 and 11 fungal isolates were obtained using the blotter and agar plate methods, respectively. The highest isolation frequency was shown by B4d and W3d which had similar morphological characteristics. Because of this findings, only B4d isolate was analysed further in this study.

Pathogenicity Test

Artificial inoculation of B4d was assessed done for pathogenicity on kenaf seeds. The fungal isolate (B4d) was found to be pathogenic in kenaf seeds. Observations of the infected seeds showed blackening and rotting of the seeds. The fungus caused more pre-emergence damping-off on the seeds (86.28%) than on the un-inoculated seeds. The isolate also reduced seed germination by 3.62% compared to un-inoculated seeds, which only reduced seed germination by 96.38%.

Cultural and Morphological Characteristics

The mycelium of the isolated fungus grow vigorously on the PDA. The aerial mycelia grew uniformly in all directions and fully covered the surface of the media within 3 to 4 days. The colour of the colony changed gradually from light grey after four to seven days of incubation (Fig. 1) to black after 2 weeks of incubation. The bottom part of the fungus

only became darker after 3 weeks of incubation. Subsequently, the fungus produced stromata and pycnidia. The pycnidia that were produced were initially soft but hardened when the culture matured at 4 weeks. The culture sporulated only after 4 weeks of incubation. A carnation leaf agar (CLA) was used to induce sporulation of the fungus. The CLA media can also be used for isolating single spore and for inducing the production of stromata and pycnidia of the fungus.

Fruiting bodies developed after 4 weeks of incubation in the CLA. Liquid exudates were produced and became dry after a few days (Fig. 2). After they were dried, the spores were released from the ostioles in the form of a black dust on the culture media. Light microscope observations showed that the fungus produced immature conidia with some distinct features. The immature conidia were non-septate, thick cell-walled, oval in shape and hyaline. However, the mature conidia showed slightly different features compared to the immature conidia. Mature conidia were observed to be septate, oval-shaped and brown in colour with the presence of irregular longitudinal striations (Fig. 3).

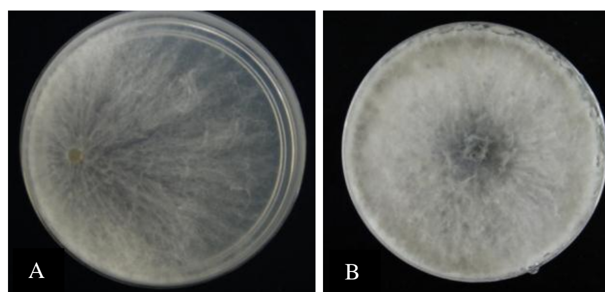

Fig. 1: Cultural characteristics of the fungus isolated from kenaf seeds. Colony was initially white and become greyish and mycelia covered the whole PDA surface. (A) Day 4; (B) Day 7

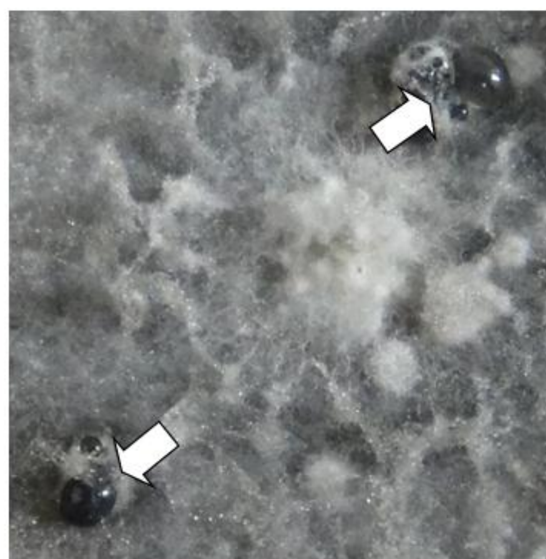

Fig. 2: B4d; developing fruiting-bodies; liquid exudates probably marking the positions of the ostioles of the pycnidia (marked by the arrow)

ITS-rDNA Sequence Analysis

The sequence was identified using BLAST and comparing data at the NCBI GenBank database. The pathogen isolated from the kenaf seeds was identified as *L. theobromae* (teleomorph: *B. rhodina*), a member of Botryosphaeriaceae, with a maximum identity and characterise of 100% (Table 2). This study represents the first effort to identify and characterise *L. theobromae* as a seed-borne pathogen in kenaf seeds. The standard PCR analysis by ITS-rDNA amplification method was used to successfully identify isolate B4d as *L. theobromae*.

A neighbour-joining tree with 1000 bootstrap replications, resulted in the division of two outgroups (i) Botryosphaeriaceae and, (ii) *Guignardia citricarpa* (Fig. 4). Outgroup (i) consisted of two subgroups (A) *Lasiodiplodia* spp. and *Diplodia*, and (B) *Fusicoccum* spp. The fungus isolate in the *Lasiodiplodia* spp. subgroup was divided into *L. crassipora* and *L. theobromae*. *L. theobromae* was always distinct from *L. crassipora* and was always located in the same subgroup of and *Botryosphaeria rhodina*, which were derived from the GenBank. The NJ phenogram resulting from the analysis was fitted into this group with a maximum identity of 100% of the test isolate. Therefore, the isolate (B4d) could be identified as *L. theobromae*.

Discussion

To our knowledge, this study is the first study to report of a seed-borne disease on *H. cannabinus* (kenaf) seeds in Malaysia. The occurrence of the fungus appeared as a greyish to black coloured colony in the PDA. This fast growing fungus was shown to be pathogenic to the seed. The pathogenicity test confirmed that the fungus caused an infection in kenaf seeds. Infected seeds showed a reduced germination percentage and death of the seeds. *Botryosphaeria* spp. is an important pathogen showing producing symptoms in many plant parts during all stages of development, including germination. Previous studies have noted that *Botryosphaeria* spp. were seed-borne pathogens and caused losses in seed germination (Sultana and Ghaffar, 2009; Owolade *et al.*, 2009).

Based on the cultural characteristics and conidial morphology of the fungus isolated from the kenaf seeds in Malaysia, the pathogen was identified as *Botryosphaeria* spp. Botryosphaeriaceae are often identified based on morphological characteristics of their associated anamorphs because their teleomorph are rare (Sivanesan, 1984; Jacobs and Rehner, 1998; Phillips, 2002). This genus of this fungus has several anamorph stages, including *Lasiodiplodia*,

Table 1: Pathogenicity of B4d on kenaf seeds at 7 days after sowing (DAS)

Treatment	Pre-emergence damping-off	Post-emergence damping-off	Germination (%)
Inoculated seeds	96.4[a]	2.4[a]	3.6[b]
Un-inoculated seeds (control)	15.0[b]	0.0[b]	85.0[a]

Means followed by the same letter are not significantly different on 95% confidence interval (P=0.05) analyzed with one way ANOVA with Duncan Multiple Range Test (DMRT)

Table 2: Identified *Lasiodiplodia theobromae* from ITS region comparison with data from GenBank

Isolate Name	GenBank Accession no.	Strain and reference	% Identity	Query length	Isolates Accession No.
B4d	FJ888469.1	CMW22664 (Mehl *et al*., 2010)	100	850	JQ809341

Dothiorella and *Fussicoccum*. These groups can be differentiated according to their conidia shape and colour (Denman *et al.*, 2000). The conidia sizes of *Diplodia*, *Dothiorella* and *Lasiodiplodia* spp. generally overlap, but *Lasiodiplodia* has slightly wider and more obovoid conidia (Burgess *et al.*, 2006). Other features that differentiate *Lasiodiplodia* spp. from other fungi in the same genus include the obvious vertical striation in their mature conidia (von Arx, 1974; Denman *et al.*, 2000). *L. theobromae* can be distinguished based on their conidia size (Burgess *et al.*, 2006).

Conidia of *L. theobromae* have been reported to be of a certain size range. In this study, the size of conidia was similar to that reported by Pavlic *et al.* (2007). The conidia were dark brown and oval shaped with a size equivalent to 24 × 15 µm (data not shown). The immature conidia were hyaline, aseptate, and oval-shaped and had thick cell walls, while the mature conidia were dark brown, septate and had thin-cell walls with vertical striations. Botryosphaeriaceae has been recorded as an important plant pathogen infecting a wide range of host plants including grapevines (van Niekerk *et al.*, 2004), *Eucalyptus* (Slippers *et al.*, 2004) and *Prunus* (Slippers and Wingfield, 2007). This pathogen has become important because it cause numerous diseases, including seed rot (Gure *et al.*, 2005), stem canker canker, dieback, root rot, fruit rot, leaf spot and witches' broom (Punithalingam, 1980). Blackening of seeds caused by *L. theobromae* has been reported to affect seed germination, viability and vigour (Nwachukwu and Umechuruba, 1996).

Botryosphaeriaceae has previously been isolated from infected plants in temperate, tropical and subtropical regions (Roux and Wingfield, 1997). According to Punithalingam (1980), *Botryosphaeria ribis*, *Botryosphaeria parva* and *Botryosphaeria dothidea* have been isolated from temperate regions, whereas *Botryosphaeria rhodina* (anamorph: *L. theobromae*) has been isolated from tropical regions. In addition to being a pathogen of woody plants, *L. theobromae* is recognised as an important seed-borne pathogen (Owolade *et al.*, 2009; Sultana and Ghaffar, 2009).

In the present study, the fungus associated with seed rot disease was isolated from kenaf seeds and identified based on cultural characteristics and observations of the anamorph fungal stage. However, molecular techniques offer the best method to identify and characterise fungal pathogens to species level. Molecular techniques also minimises the confusion due to overlap of morphological characteristics (Pavlic *et al.*, 2004; Burgess *et al.*, 2006). The genus *Botryosphaeria* was differentiated using DNA sequence data (amplification of rDNA ITS 1 and ITS 4) derived from fungal species (Slippers *et al.*, 2004; Begoude *et al.*, 2010).

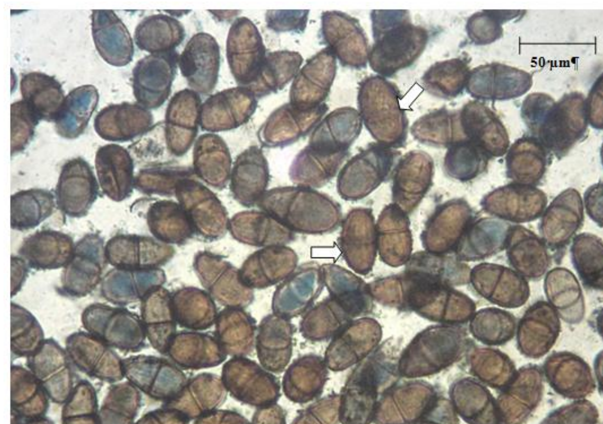

Fig. 3: Conidia was observed under light microscope. Mature conidia with thin cell wall, oval-shaped and dark brown colour (marked by arrows)

Amplification of the ITS-rDNA region provided the best results for identifying and describing the fungus. A sequence comparison with the NCBI database confirmed that the fungus was *L. theobromae*. The sequence data allowed for the verification of the *L. theobromae* isolate from *H. cannabinus* grown in a tropical area.

This study showed that ITS rDNA sequence data can be used to distinguish Botryosphaeriaceous species. Despite, the general phylogenetic usefulness of this region of the genome, there are cryptic species that cannot be separated based solely on their ITS-rDNA sequence data (De Wet *et al.*, 2003; Slippers *et al.*, 2004). Thus, a combination of sequence data and morphological characteristics is beneficial for clarifying the taxonomy of this pathogen.

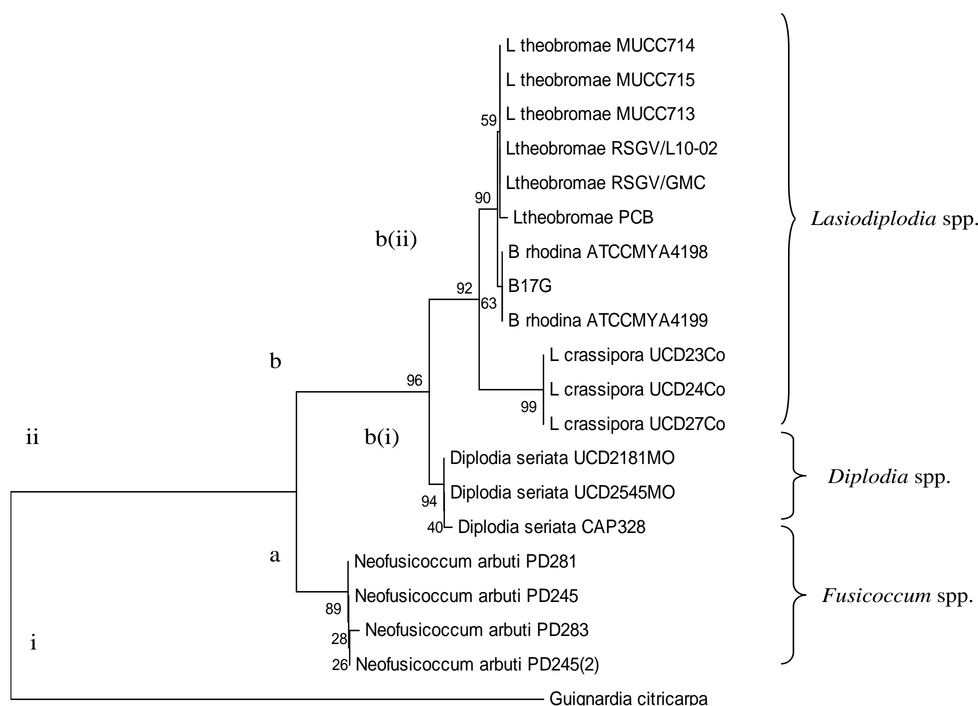

Fig. 4: Neighbor-Joining tree based on ITS-rDNA gene sequence similarity showing the phylogenetic position of the fungal pathogen obtained from kenaf seeds. Boostrap analysis was made with 1000 cycles. Bar scale, 0.05 substitutes per nucleotide position

Conclusion

The seed-borne fungal pathogen of kenaf was identified as *L. theobromae* based on its cultural, morphological and molecular characteristics. Although, the pathogen can reduce seed germination, the infection has not reached a critical stage. Therefore, precautionary action is needed to prevent any future outbreaks of this disease.

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